Short chain chito-oligosaccharides promote arbuscular mycorrhizal colonization in Medicago truncatula

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Abstract: During the establishment of arbuscular mycorrhizal (AM) symbiosis, the fungus and the host plant exchange chemical signals that are crucial to reciprocal recognition. Short-chain chitin oligomers (CO) released by AM fungi are known to trigger symbiotic signaling in all host plant species tested. Here we applied exogenous CO, derived from crustacean exoskeleton, to pot-grown Medicago truncatula inoculated with the AM fungus Funneliformis mosseae and investigated root colonization, plant gene regulation and biomass production. CO treatment strongly promoted AM colonization with significant increases in arbuscule development, biomass production and photosynthetic surface compared to untreated mycorrhizal plants. Gene expression analyses indicated that CO treatment anticipated the expression of MtBCP and MtPT4 plant symbiotic markers, during the first two weeks post inoculation. Altogether, our results provide evidence that plant treatment with symbiotic fungal elicitors, anticipated and enhanced AM development, encouraging the use of CO to promote AM establishment in sustainable agricultural practices.

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Cover Letter

Torino, 30.7.2019

Dear Editor,

We are submitting the manuscript entitled «*Short chain chito-oligosaccharides promote arbuscular mycorrhizal colonization in Medicago truncatula*» to be considered for publication in Carbohydrate Polymers**.**

Over 80% of plants, including most crops, live in a mutualistic symbiotic association, called arbuscular mycorrhiza (AM), with an ancient group of soil fungi that provide their hosts with soil water and mineral nutrients in change for sugars and lipids. For this key role in plant nutrition, the introduction of AM as biofertilizers in **sustainable agricultural practices** has been proposed for decades as a promising natural solution to the increasing worldwide demand of food safety. Nevertheless, field applications tests have delivered contrasting results, and experimental data indicate a partial recalcitrance of cultivated species toward AM interaction, likely due to decades of excessive use of fertilizers and to farming practices that have been unaware of the plant underground interactions.

We now know that plant-fungus recognition is an essential step in AM establishment and is based on an exchange of chemical signals: root-exudates alerts the AM fungus of host proximity, while AM fungi release **short-chain chito-oligosaccharides** (CO) eliciting the plant symbiotic responses, such as gene regulation, starch reallocation and cell rearrangement for fungal accommodation.

In the light of this role for CO as AM fungal signals, we decided to **test whether their exogenous application could have a positive impact on symbiosis establishment**. This would on the one hand demonstrate unambiguously the biological role of CO as symbiosis elicitors and, on the other hand, open the way to their use as promoters of AM colonization in crops for sustainable, food safety-oriented agricultural applications.

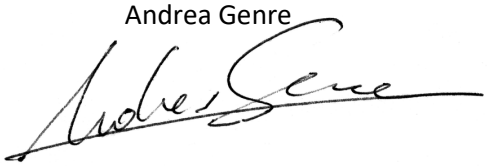
To this aim, we treated the model legume *Medicago truncatula* with a mixture of short-chain CO (ranging between 2 and 5 N-acetyl-glucosamine residues) and observed how their application influenced plant mycorrhizal status and development.

Indeed, our analyses of root colonization intensity, plant gene expression and biomass development consistently showed that **CO treatments strongly enhance and anticipate AM colonization**. This represents a **breakthrough** on the way to the large-scale introduction of AM in agricultural practices, by positively acting on the mycorrhizal aptitude of the host plants.

For these reasons we consider that the findings in this article are of primary interest in the field of carbohydrate polymer applications to plant-microbe interactions, and appropriate for timely publication in your Journal.

Yours sincerely

Andrea Genre



Highlights (for review)

- Arbuscular mycorrhizal (AM) symbiosis supports life of most crop plants.
- The fungus is recognized as a symbiont via water-soluble short-chain chitin oligomers (CO)
- We tested whether exogenous CO application to *Medicago truncatula* could impact on the symbiosys.
- CO treatment strongly promoted AM colonization with significant increases in arbuscule development, biomass production and photosynthetic surface compared to untreated mycorrhizal plants.
- Plant treatment with symbiotic fungal elicitors anticipated and enhanced AM development, encouraging the use of CO to promote AM establishment in sustainable agricultural practices

Short chain chito-oligosaccharides promote arbuscular mycorrhizal colonization in *Medicago truncatula*

Authors

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Abstract

During the establishment of arbuscular mycorrhizal (AM) symbiosis, the fungus and the host plant exchange chemical signals that are crucial to reciprocal recognition. Short-chain chitin oligomers (CO) released by AM fungi are known to trigger symbiotic signaling in all host plant species tested. Here we applied exogenous CO, derived from crustacean exoskeleton, to pot-grown *Medicago truncatula* inoculated with the AM fungus *Funneliformis mosseae* and investigated root colonization, plant gene regulation and biomass production. CO treatment strongly promoted AM colonization with significant increases in arbuscule development, biomass production and photosynthetic surface compared to untreated mycorrhizal plants. Gene expression analyses indicated that CO treatment anticipated the expression of *MtBCP* and *MtPT4* plant symbiotic markers, during the first two weeks post inoculation. Altogether, our results provide evidence that plant treatment with symbiotic fungal elicitors, anticipated and enhanced AM development, encouraging the use of CO to promote AM establishment in sustainable agricultural practices.

1. Introduction

The life of most plants, including the majority of crops, is supported by a mutualistic root symbiosis with Glomeromycotina, an ancient group of soil fungi (Spatafora et al., 2016) that provide their hosts with a preferential access to soil water and mineral nutrients, while taking advantage of plant-photosynthesized sugars and lipids (Smith & Read, 2008; Wewer et al. 2014; Keymer et al., 2017).

Plant-fungus recognition is essential for AM establishment and is based on an exchange of chemical signals (Bonfante & Requena, 2011; Zipfel & Oldroyd, 2017). Root-exuded strigolactone alerts the AM fungus of host proximity (Akiyama et al., 2005); its perception activates spore germination, hyphal metabolism and branching (Besserer et al., 2006). In turn, AM fungi release diffusible molecules eliciting the plant symbiotic response (Maillet et al, 2011; Genre et al., 2013). These include local and systemic changes in gene expression (Kosuta et al., 2003), root-directed starch reallocation (Gutjahr et al., 2009), and the activation of a signal transduction pathway, partially shared with other symbiotic interactions (Oldroyd, 2013; Delaux et al., 2015; Barker et al., 2017; Genre and Russo, 2016). This pathway includes the triggering of repeated oscillations (spiking) in nuclear calcium concentration, making the detection of nuclear Ca^{2+} spiking an acknowledged benchmark for the induction of symbiotic responses in AM hosts.

Indeed, Ca^{2+} spiking is observed in root epidermal nuclei upon adhesion of AM fungal hyphopodia (Chabaud et al., 2011), application of AM fungal exudate (Navazio et al, 2007; Chabaud et al., 2011; Genre et al., 2013) or purified molecules isolated from fungal exudate, such as short-chain chito-oligosaccharides (CO) and lipo-chito-oligosaccharides (LCO) (Maillet et al., 2011; Genre et al., 2013). In particular, fungal release of tetrameric and pentameric CO is boosted upon strigolactone treatment (Genre et al., 2013). Furthermore, their activity has been demonstrated in legumes (Genre et al., 2013), carrot (Genre et al., 2008), rice (Sun et al., 2015; Carotenuto et al., 2017), *Discaria trinervis* and *Casuarina glauca* (Chabaud et al., in press).

In the light of this universal role for CO in eliciting early plant responses to AM fungi (Genre &

Russo, 2016), we decided to test whether their exogenous application could have a positive impact on symbiosis establishment. This would on the one hand demonstrate the biological role of CO as symbiosis elicitors and, on the other hand, open the way to their use as promoters of AM colonization in crops for sustainable, food safety-oriented agricultural applications (Berruti et al., 2015).

To this aim, we treated the model legume *Medicago truncatula* with a mixture of short CO (ranging below 6 N-acetyl-glucosamine residues), obtained from crustacean manufacturing industry, and observed how their application influenced plant development and mycorrhizal status.

2. Materials and Methods

2.1. Plant growth and mycorrhization.

Seeds of *M. truncatula* (cv. Jemalong, genotype A17) were scarified for 3 min in H_2SO_4, rinsed several times in distilled water, sterilized for 2 min in diluted commercial bleach (1:5), rinsed 4 times in sterile distilled water and pre-germinated for 48 hours on agar plates (0.6% w/v) at 23° C in the dark. Seedlings were grown for ten days in a photoperiod of 16h day (23°C) and 8h night (21°C) before transferring them to 10x10x12cm pots containing quartz sand.

The AM fungus *Funneliformis mosseae* (strain BEG 12) was inoculated in mycorrhizal pots by replacing 10% of the pot substrate with a commercial inoculum (MycAgroLab, Bretenière, France) and mixing. All plants were watered once a week with half-strength Long-Ashton nutrient solution containing 3,2 µM KH_2PO_4 as P source (Hewitt, 1966).

2.2. Chito-oligosaccharides

We used a commercial mixture of short chain CO purified from crustacean manufacturing side-products (Zhengzhou Sigma Chemical Co., Ltd., Henan, China). *HPLC-MS/MS* analysis of the CO mixture (File S1) confirmed the presence of deacetylated, mono-acetylated and di-acetylated CO molecules composed of 2 to 5 N-acetyl-glucosamine residues (Fig. S1).

76

77 *2.3. Chito-oligosaccharide solutions*

78 1g/L and 1mg/L CO solutions in sterile distilled water were tested for the triggering of Ca^{2+} spiking
79 in the root epidermis. Only the 1g/L solution was chosen for pot treatments, to counteract molecule
80 dilution/absorption and compensate for the presence of biologically inactive CO2 and CO3 in the mix,
81 alongside the AM signals CO4 and CO5 (Fig. S1). CO application to pot-grown plants was done
82 either by irrigating the substrate with the CO solution, or by spraying the solution on plants and soil
83 surface.

84

85 *2.4. Analysis of CO-induced Ca^{2+} signals*

86 Confocal microscopy was performed on 2cm-long lateral roots of *Agrobacterium rhizogenes*-
87 generated root organ cultures (ROC) of *M. truncatula* expressing NUP-YC2.1, a nuclear localized
88 Ca^{2+}-sensing yellow cameleon construct (Chabaud et al., 2011). Detection and plotting of relative
89 changes in Ca^{2+} concentration in epidermal atrichoblasts was done according to Genre et al. (2013),
90 as described in File S1. At least 100 atrichoblasts from seven independent root samples were analyzed
91 for each condition and the t-Student test with a probability level of $P < 0.05$ was used for statistical
92 validation.

93

94 *2.5. Plant treatment with CO*

95 In order to evaluate CO effect on plant development and mycorrhization, four experimental
96 conditions were set up: control (Ctr), lacking both CO treatment and AM inoculation; CO-treated
97 control (Ctr+CO); mycorrhizal (Myc), where AM inoculum was added in the absence of CO
98 treatments; and CO-treated mycorrhizal (Myc+CO), where plants were both inoculated and exposed
99 to the CO solution.

100 For CO application by irrigation, residual water was carefully removed from the pot saucers before

watering each Ctr+CO and Myc+CO pot with 30 mL of CO solution. Distilled water was used for Ctr and Myc pots. Treatments were applied weekly, and plants were sampled 48 days post inoculation (dpi), after six CO treatments (Fig. S2), for morphometric analyses and mycorrhizal intensity determination.

For spray treatments, 5 mL of CO solution containing 0.005% Tween20 as a surfactant, was sprayed weekly over Ctr+CO and Myc+CO pots from a 10 cm distance, using plastic shields to confine spray diffusion. Ctr and Myc pots were sprayed with 5 mL of 0.005% Tween20 in sterile distilled water. Plants treated with CO spray were divided in two groups (Fig. S2): one was grown for 28 dpi (with two CO treatments) and the other for 48 dpi (with six CO treatments) before measuring their morphological and physiological parameters and quantifying AM colonization.

2.6. *Analyses of plant development after CO treatment*

Irrigation-treated plants were harvested at 48 dpi; root and shoot fresh weight was immediately measured. To determine the leaf area, green tissues were carefully isolated and placed on an Epson Perfection V800 Photo scanner, to obtain black and white scanned images of the leaf surface at 600 dpi resolution. The resulting PNG images were then analyzed using Fiji/ImageJ (http://imagej.nih.gov/;http://fiji.sc/Fiji) to calculate total leaf surface in mm^2. Following image acquisition, shoot dry weight was measured after exsiccation at 60°C for 24h.

Spray-treated plant growth was monitored (Fig. S3) by photography at 7 dpi (prior to CO treatment), 28 dpi (two treatments) and 48 dpi (six treatments). Five Ctr, five Ctr+CO, ten Myc and ten Myc+CO plants were harvested at 28 dpi and 48 dpi, to measure their fresh and dry weight and photosynthetic surface, as described above.

2.7. Nitrogen balance index

The physiological status of spray-treated plants was estimated before sampling using a Dualex 4

126 (FORCE-A, Orsay, France) to determine flavonoid and chlorophyll content by measuring leaf UV
127 absorbance and fluorescence, respectively, in 10 randomly chosen leaves from 5 Ctr and 5 Ctr+CO
128 plants, and 20 leaves from 10 Myc and 10 Myc+CO plants. Measures were done on both adaxial and
129 abaxial leaf surface and the two values were averaged (Cartelat et al., 2005). The
130 chlorophyll/flavonoid ratio, or Nitrogen Balance Index (NBI), is considered an indicator of the plant
131 nitrogen nutritional status (Cerovic et al., 2012).
132
133 *2.8. Quantification of mycorrhizal colonization*
134 Following fresh weight measure, each root system was stained with 0.1% cotton blue in lactic acid
135 and quantitative parameters of mycorrhizal intensity were calculated as described by Trouvelot et al.
136 (1986), using the online tool MYCOCALC (http://www2.dijon.inra.fr/mychintec/Mycocalc-
137 prg/download.html).
138
139 *2.9. Gene expression analysis following CO treatment*
140 To investigate the ability of CO treatment to stimulate host receptiveness for AM fungi, a time-
141 course experiment was set up where plants received two rounds of spray treatment before AM
142 inoculation, 21 and 23 days post transplant (dpt). The inoculum was added at 25 dpt, and plants were
143 sampled at 7, 10, 14, 21 and 28 dpi. Five leaf replicates per treatment were sampled to analyse
144 phosphorus (P) content and five root replicates from Myc and Myc+CO were sampled and frozen in
145 liquid nitrogen for gene expression analyses.
146
147 *2.10. RNA isolation and quantitative RT-PCR analysis*
148 Total RNA was isolated from roots, using the RNeasy™ Plant Mini kit (Qiagen, Hilden, Germany).
149 Samples were disrupted with a TissueLyser (Qiagen Retsch GmbH, Hannover, Germany) and treated
150 following manufacturer instructions. RNA quality and quantity were evaluated using a Nanodrop and

agarose gel electrophoresis.

To remove genomic DNA contamination, RNA samples were digested with TURBO™ DNase (Ambion) according to manufacturer instructions and checked for DNA traces by conventional PCR using *MtTEF* primers, before cDNA synthesis and qRT-PCR analyses (File S1). The sequences of all genes studied are listed in Table S1.

2.11. Assessment of arbuscule morphology

Root colonized segments from Myc and Myc+CO plants were embedded in agarose (8%) and cut into 200 µm-thick sections using a Vibratome (Oxford Vibratome®). Sections were collected on microscope slides and stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC; Sigma-Aldrich, Milan, Italy), as described in Volpe et al. (2016). Twenty root segments from five biological replicates were analyzed for each treatment.

2.12. Phosphorus quantification

Phosphorus quantification was done in 5 Ctr, 5 Ctr+CO, 10 Myc and 10 Myc+CO plants, by collecting the third leaf from the tip of the main stem. Collected leaves were lyophilized, weighed and digested in 1 mL of 6M HNO_3 for one hour at 95°C. The digestion product was diluted in 6 mL of sterile distilled water. A control solution, without any sample, was treated with the same procedure. All solutions were analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) to determine P content.

2.13. GenBank accession numbers

AY116211 (*MtPT4*); XM_003592405.2 (*MtBCP*); XM_013595882.1 (*MtTEF*); XM_013606824 (*MtPR10*); XM_003604044.2 (*MtChit1*); XM_003618769.2 (*MtChitIII.1*); XM_003597718.2 (*MtChitIII.2*); AY238969.2 (*MtChitIII.3*); AY238970.1 (*MtChitIII.4*)

3. Results

3.1. Crustacean-derived CO triggered Ca^{2+} spiking in M. truncatula atrichoblasts

In order to assess the commercial CO effectiveness as symbiotic elicitors, we recorded nuclear Ca^{2+} signals in atrichoblasts of NUP-YC2.1 *M. truncatula* ROCs treated with 1g/L and 1mg/L CO solutions (Fig. 1). Both solutions triggered comparable spiking patterns, with frequent, well-defined peaks, in analogy with those induced by analogous concentrations of purified CO4, CO5 or raw AM spore exudate (Chabaud et al., 2011; Genre et al., 2013). As expected, no spiking was recorded upon control treatment with sterile distilled water. The percentage of responding atrichoblasts and average peak numbers confirmed the lack of statistically significant differences in the cell response to either CO concentration (Fig. 1B).

In conclusion, our bioassay confirmed that both 1g/L and 1mg/L solutions of the commercial CO mix was able to mimic AM fungal signals in triggering Ca^{2+}-mediated symbiotic signaling.

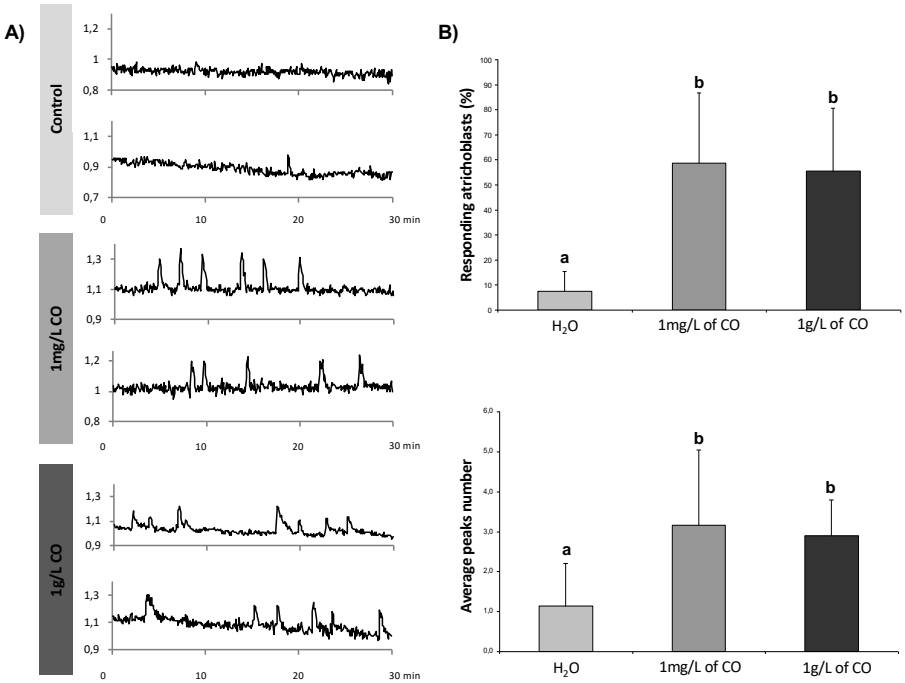

Fig. 1. Nuclear Ca^{2+} spiking in response to crustacean-derived CO. A) Representative plots of nuclear Ca^{2+} variations over 30 minutes in atrichoblast nuclei of *M. truncatula* root organ culters (ROCs) treated with water (control), 1mg/L and 1g/L CO. Both CO concentrations induced a series of Ca^{2+} peaks (spiking), characteristic of AM fungal recognition. B) Statistical analyses of the spiking response (t-Student test with $P < 0.05$) did not highlight significant differences between CO treatments, in terms of either the percentage of responding atrichoblasts (top panel) or average peak number in responding cells (bottom). Bars represent standard errors. At least 100 atrichoblasts from seven independent root samples were analyzed for each experimental condition.

3.2. CO application by irrigation boosted AM colonization

In a first attempt to investigate the effect of exogenous CO supply, we grew inoculated and non-inoculated *M. truncatula* in pots, with or without a weekly supplement of CO in the watering solution, and analyzed their biomass, photosynthetic surface and mycorrhizal colonization at 48 dpi. While root development was comparable in Ctr, Myc and Myc+CO plants, statistically significant differences were observed in the shoot fresh and dry biomass between Ctr and both mycorrhizal conditions, Myc and Myc+CO (Fig. S4). The same trend was confirmed for photosynthetic surface, with similar top values for Myc and Myc+CO plants and a significantly smaller area for Ctr and Ctr+CO plants. Remarkably, Ctr+CO plants showed a significant reduction in biomass and photosynthetic surface, when compared to all other treatments, suggesting that CO perception in the absence of fungal inoculation could induce a stress condition inhibiting plant growth.

AM colonization was then assessed in Myc and Myc+CO plants through optical microscopy. Isolated groups of arbuscules were distributed along the root axis of Myc plants, spaced by uncolonized cortical cells (Fig. S5). By contrast, Myc+CO plants displayed a more extensive and homogeneous distribution of arbuscules, with a continuous mass of arbusculated cells, which also involved multiple layers of the root cortex (Fig. S5). This was confirmed by our quantitative analysis, with significantly higher frequency (F), intensity of mycorrhization (M) and arbuscule abundance (a, A) in Myc+CO compared to Myc plants (Fig. S5).

In conclusion, beside a partial growth inhibition in uninoculated plants, exogenous CO application by irrigation strongly improved the mycorrhizal status of AM inoculated plants at 48 dpi.

3.3. CO spray treatment enhanced AM colonization

These encouraging results, coupled with the possible induction of a stress response in Ctr+CO, prompted us to test an alternative treatment. We chose spray application as an amenable method of CO supply in view of possible field treatments. For these experiments, we sampled our plants at 28

and 48 dpi to obtain a clearer picture of symbiosis development.

Our first focus was to check whether the spray treatment could be as efficient in promoting AM development as the irrigation method. Indeed, F, M and A values were all significantly higher in Myc+CO compared to Myc plants at 28 dpi (Fig. 2). Also in this case, CO treatment caused arbuscule proliferation across multiple cortical layers, compared to the fewer arbuscules, with spotted distribution, found in Myc plants. At 48 dpi, all mycorrhization parameters surged in both conditions, reducing the difference between Myc and Myc+CO plants, which anyway remained significant (Fig. 2).

In short, the quantification of AM colonization based on microscopic observations confirmed a positive effect on mycorrhizal intensity also for CO spray treatment.

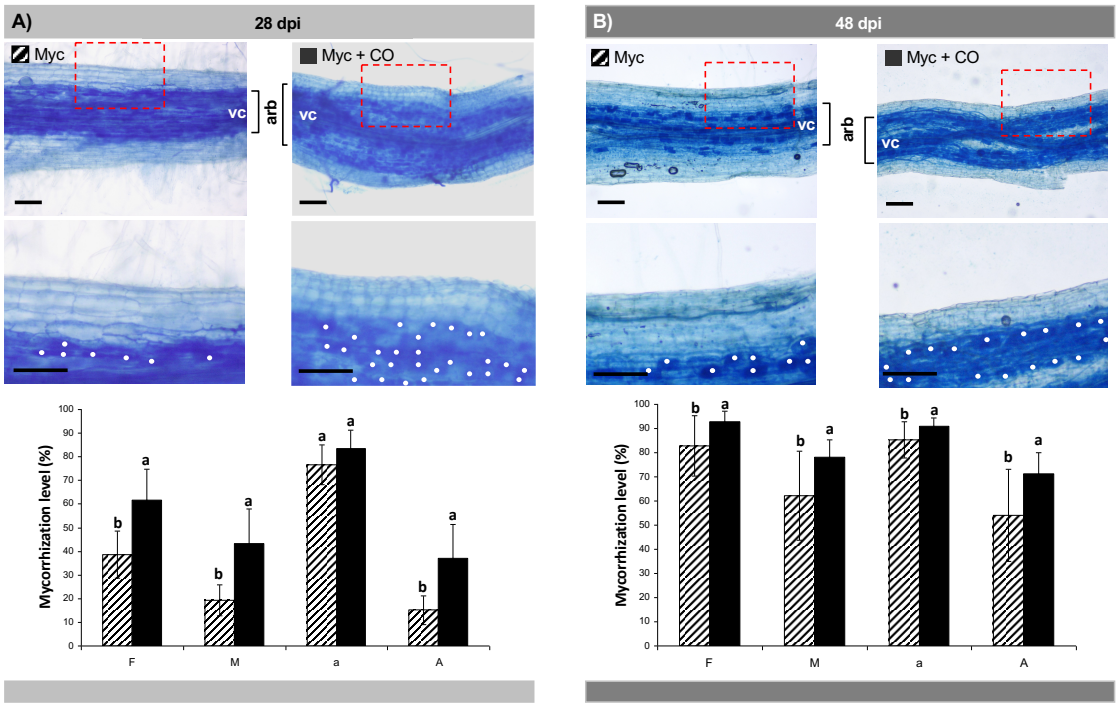

Fig. 2. Effect of spray CO treatment on mycorrhizal plants at 28 dpi (A) and 48 dpi (B). Images of root sections of mycorrhizal plants untreated (Myc) and treated with CO (Myc+CO). A strong proliferation of arbuscules (white dots) was evident in Myc+CO plants, with the colonization of multiple cortical layers at both time points, as evident in the magnified areas (red square) in the lower panels. This was confirmed by the quantitative analysis of root colonization, shown in the bottom graphs: mycorrhization frequency (F), mycorrhization intensity (M), arbuscule abundance within colonized root fragments (a) and arbuscule abundance in the whole root system (A) all reported significantly higher values in Myc+CO compared to Myc plants. The effect of CO on root colonization was anyway more evident at 28 dpi. Mean values and SDs of ten biological replicates for each treatment are shown. Bars = 100 µm.

3.4. CO spray treatment and AM inoculation synergistically increase plant growth

Photographic monitoring of shoot growth showed that prior to any CO treatment - at 7 dpi - all plants had a comparable development (Fig. S3). By contrast, significant differences became evident at 28 dpi (two CO treatments) and even more pronounced at 48 dpi (six CO treatments): at both time points, Myc and Myc+CO plants were larger than Ctr plants. Interestingly, CO application by spray to uninoculated plants (Ctr+CO) did not affect their growth at any time point (Fig. S3), in contrast with the results of CO treatment by irrigation.

Such macroscopical observations were partially confirmed by biometric measurements. At 28 dpi (Fig. 3A), the photosynthetic surface was significantly more extensive in mycorrhizal treatments compared to both Ctr and Ctr+CO, with the highest value for Myc+CO plants, albeit not significantly larger than in Myc plants. An analogous trend was recorded for shoot dry biomass, with the highest values for the Myc and Myc + CO plants, even if differences were not statistically significant.

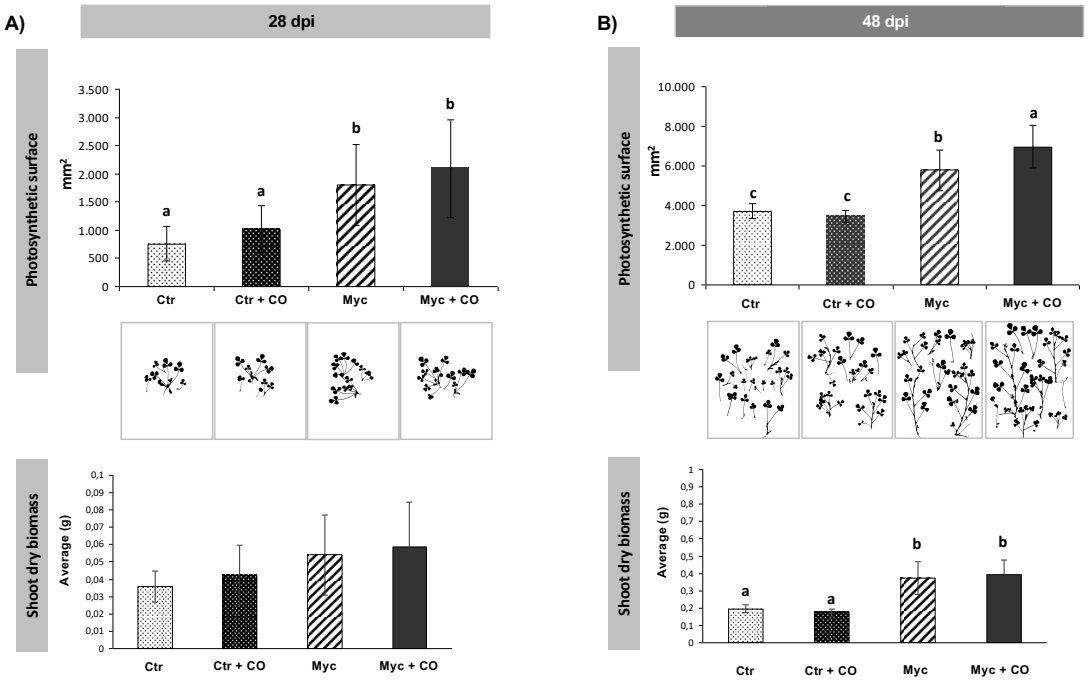

Fig. 3. Aboveground development in response to spray CO treatment. A) Average photosynthetic surface (including representative scans of the plant epigeral parts) and shoot dry biomass at 28 dpi: the photosynthetic surface was significantly more developed in Myc and Myc+CO plants compared to Ctr and Ctr+CO; a similar – albeit statistically non significant - trend was observed for shoot dry weight. B) The photosynthetic surface was significantly more development in Myc+CO plants at 48 dpi, when both mycorrhizal plants showed a significant increase of shoot dry biomass. Mean values and SDs are presented for five and ten biological replicates from control and mycorrhizal plants, respectively.

241 At 48 dpi (Fig. 3B), both mycorrhizal conditions (Myc and Myc+CO) showed a significantly larger
242 photosynthetic surface than both Ctr and Ctr+CO, but in addition, Myc+CO significantly outclassed
243 Myc plants at this later time point. An analogous significant increase was observed in shoot dry
244 biomass between mycorrhizal and uninoculated plants, albeit Myc and Myc+CO had very similar
245 values.

246 In conclusion, two CO spray treatments were sufficient to induce a significant increase in AM
247 colonization at 28 dpi, while a CO-dependent increase in plant development was evident at 48 dpi,
248 after six CO treatments.

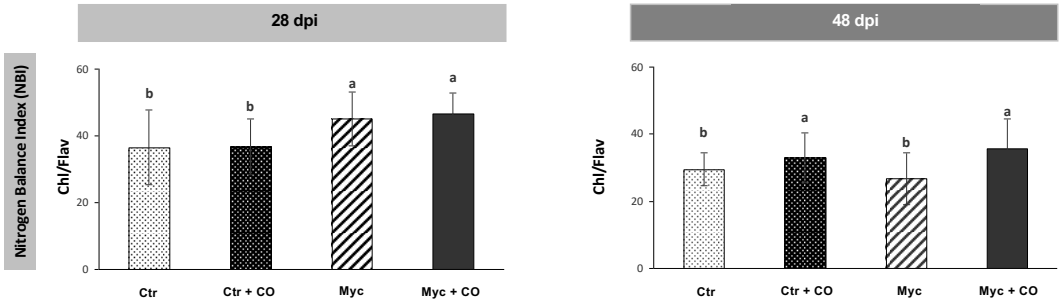

Fig. 4. Leaf nitrogen (N) status at 28 and 48 dpi. The chlorophyll/flavonoid ratio (Chl/Flav), or Nitrogen Balance Index (NBI), is presented here as an indicator of leaf N content. NBI value was significantly higher in both mycorrhizal lines (Myc and Myc+CO) than controls (Ctr and Ctr+CO) at 28 dpi. By contrast, NBI was significantly higher in all CO treated plants at 48 dpi. Mean values and SDs are presented for five and ten biological replicates from control and mycorrhizal plants, respectively.

249

250

251 *3.5. Effect of CO spray on plant nitrogen status*

252 We measured the nitrogen balance index (NBI) to indirectly monitor plant nitrogen status and
253 overall stress. At 28 dpi, the NBI was significantly higher for both mycorrhizal conditions, indicating
254 a better metabolic performance compared to Ctr and Ctr+CO plants (Fig. 4). No significant difference
255 was observed between the latter, confirming that CO spray alone did not cause significant stress in
256 uninoculated plants. By contrast, at 48 dpi, the NBI was significantly higher in CO-treated plants
257 (Ctr+CO and Myc+CO) compared to Ctr and Myc.

258 Altogether, NBI analysis indicated a comparable increase in leaf nitrogen content for both

mycorrhizal conditions at 28 dpi, and a further increase in Myc+CO at 48 dpi, in line with CO effects on plant development.

3.6. Plant AM marker genes are regulated in response to CO treatment

Since CO supply had effectively enhanced root colonization at 28 and 48 dpi, we decided to investigate in more detail the effect of CO with a time-course experiment that included earlier time points. In addition, CO ability to stimulate host plant receptiveness for AM fungi was assessed by spraying plants twice with the CO, before fungal inoculation (Fig. S2). We consequently analyzed gene expression for two established AM markers: *MtBCP* (*Blue Copper-binding Protein*), expressed during arbuscule development, and *MtPT4* (*Phosphate Transporter 4*), encoding an AM-specific P transporter localized on the periarbuscular membrane of active, mature arbuscules (Pimprikar & Gutjahr, 2018).

MtBCP expression was more strongly induced in Myc+CO plants at 7, 10 and 14 dpi, compared to a later activation in Myc plants (Fig. 5), with a significant difference at 7 dpi. A drastic downregulation was recorded at 21 and 28 dpi, in line with the expected reduction of *MtBCP* expression in terminal stages of root colonization. Similarly, *MtPT4* was also activated earlier in Myc+CO plants, but its expression surprisingly decreased at 21 dpi, apparently anticipating the analogous decrease observed in Myc plants at 28 dpi.

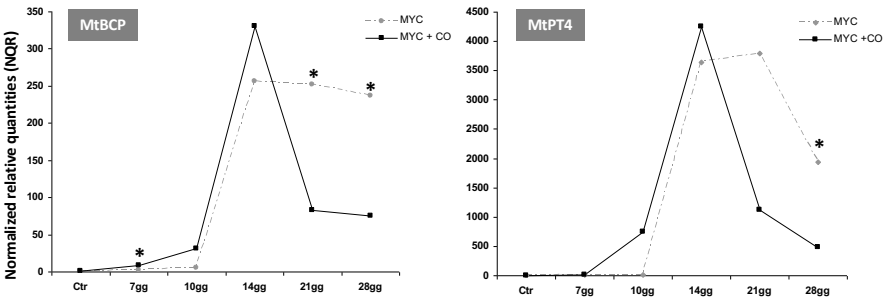

Fig. 5. Time-course analysis of *MtBCP* and *MtPT4* expression. Asterisks indicate the significant early induction of *MtBCP* at 7 dpi and the significant downregulation of both genes at 21 and 28 dpi in CO-treated plants (Myc+CO). Average values from at least 5 biological replicates are presented.

To better investigate this unexpected difference in AM marker gene expression, we anayzed the

regulation of three AM-induced chitinases (Salzer et al, 2000; Kremer et al., 2013; Malolepszy et al, 2018) in fully developed mycorrhizal roots, at 28 dpi: *MtChitIII.2*, *MtChitIII.3* and *MtChitIII.4*. In our hands, only *MtChitIII.2* and *MtChitIII.4* were upregulated in inoculated plants compared to controls (Fig. 6), but both chithinases were less expressed in Myc+CO compared to Myc plants. In conclusion, two additional markers of arbuscule maturity displayed the same downregulation as *MtPT4* in Myc+CO plants.

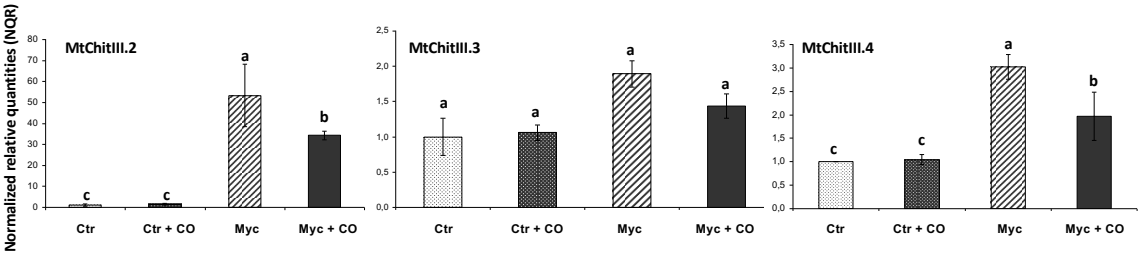

Fig. 6. Molecular analysis on roots at 28 dpi after two CO treatments. Two of the three AM-induced chitinases are strong induced in the mycorrhizal plants, but the transcripts levels for *MtChitIII.2* and *MtChitIII.4* are significant reduced in the Myc+CO compared to the Myc untreated plants, in line with the expression profile of *MtPT4*. Mean values and SDs in three biological replicates for each treatment are shown.

On this basis, we wondered whether this pattern of gene regulation could be indicative of anticipated arbuscule degeneration in CO-treated mycorrhizal plants - an aspect that could not be highlighted by our quantitative analyses of mycorrhizal colonization. We therefore compared arbuscule morphology in Myc and Myc+CO plants (Fig. S6). No obvious difference was observed in arbuscule shape and general organisation: the expected arbuscule morphology, with a large trunk and a mass of fine branches occupying most of the host cell volume, was consistently recorded for both conditions, suggesting that the vast majority of arbuscules was fully developed, independent of CO application.

Altogether, CO treatment on the one hand accelerated root colonization by the AM fungus, providing a convincing demonstration of CO role as elicitors of the host plant symbiotic response. Exogenous CO application led to an anticipated expression of early AM markers such as *MtBCP* and the development of a higher number of arbuscules that appeared functional, based on their morphological features. On the other hand, CO treatment reduced the late expression of AM marker genes that are normally expressed in active arbusculated cells (Harrison et al., 2002; Elfstrand et al.,

2005; Pumplin & Harrison, 2009). This unexpected result prompted us to compare the nutritional status of Myc and Myc+CO plants.

3.7. Leaf phosphorus content was not affected by CO treatment

We compared P content in leaf samples from both mycorrhizal conditions. Surprisingly, no significant difference was detected for any time point: plants reached their highest P content at 14 dpi under both conditions (Fig. 7), with a progressive decrease in the following time points.

In conclusion, the increase in arbuscule abundance in Myc+CO plants was not reflected by an improvement in plant P uptake under our experimental conditions.

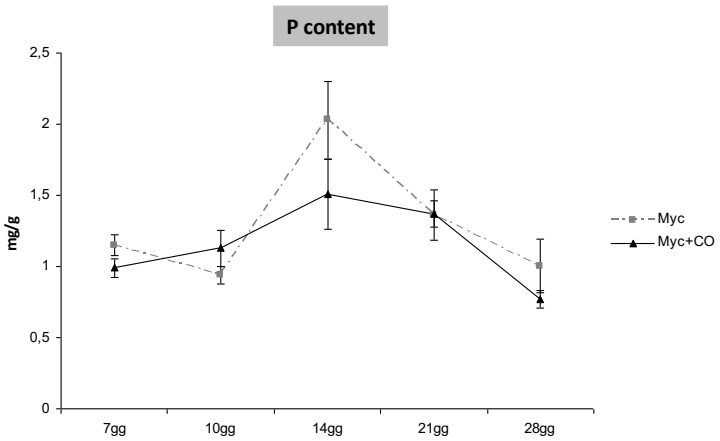

Fig. 7. Phosphorous (P) content level in untreated (Myc) and treated (Myc+ CO) mycorrhizal plants. Both lines reached the highest leaf P content at 14 dpi but no significant difference was detected in any time point. Mean values for five biological replicates are shown. Bars represent standard errors.

3.8. CO spray does not upregulate plant defense markers

To have a more comprehensive view of CO effects on the plant, we also checked whether CO perception could activate plant defense responses, by analyzing the expression of the pathogenesis related gene *MtPR10* and two pathogenesis-related chitinases, *MtChitI* and *MtChitIII.1* (Salzer et al., 2000; Lipka et al., 2005). None of these defense markers was induced by CO treatment (Fig. 8), in line with literature data about the scarce activity of short chain CO as defense elicitors (Shibuya & Minami, 2001). Moreover, the significant reduction of *MtChitI* expression in Ctr+CO, Myc and Myc+CO plants compared to Ctr, suggests a possible partial inhibition of defense responses in the

317 presence of either fungal or externally supplied (or both) signals.

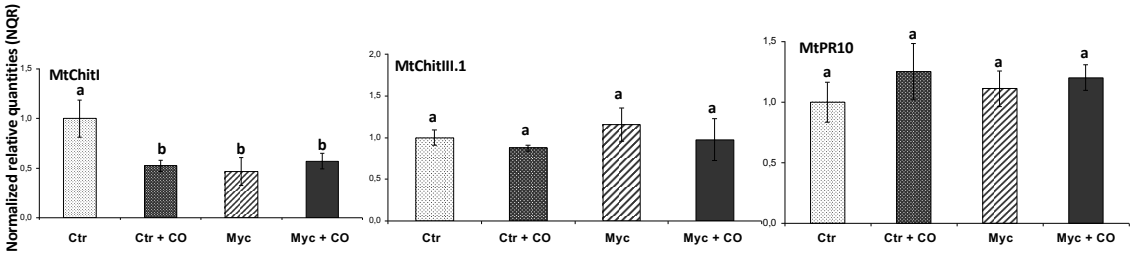

Fig. 8. Expression of pathogenesis-related genes in roots at 28 dpi after two CO treatments. The three analysed pathogenesis-induced genes, *MtChitI*, *MtChitIII.1* and *MtPR10*, were not upregulated upon treatment with crustacean-derived CO, suggesting that they were not perceived as a pathogenesis signal. Mean values and SDs in three biological replicates for each treatment are shown.

318

319 **4. Discussion**

320 In spite of several consistent reports of CO role as elicitors of early AM signaling and a few
321 investigations of CO-regulated gene expression (Weidmann et al, 2004; Giovannetti et al, 2015; Sun
322 et al., 2015), surprisingly few studies have investigated the effect of CO on symbiosis development
323 (Ramírez et al., 2010). In the light of our results, CO now appear particularly promising for large
324 scale agricultural applications for several reasons: among currently characterized AM fungal signals,
325 CO can be obtained at a fraction of the cost of the more complex LCO (lipo-chito-oligosaccharides,
326 similar to rhizobial Nod factors), from waste products of crustacean fishing industry. As natural and
327 easily bio-degradable products, CO are considered environmentally friendly and the use of chitin-
328 derived molecules has a relatively long history in agriculture, for their stimulation of plant growth
329 and defense (Benhamou et al., 1998; Sharp, 2003; Zhang et al., 2016; Winkler et al., 2017).

330

331 *4.1. Exogenous CO application promotes AM development*

332 The most striking result of this study was the observation that CO treatment induced a remarkable
333 increase in arbuscule abundance. This evidence represents a crucial advance in support of the role of
334 CO as positive elicitors of symbiotic responses in the host plant, demonstrating their effect - beyond
335 early signaling and gene expression – on the development of arbuscules, the functional core of AM
336 symbiosis.

In addition, our results highlighted that spraying the plants with CO prior to AM fungal inoculation anticipated the upregulation of *MtBCP* and *MtPT4* at 7 and 10 dpi, suggesting the ability of CO to accelerate AM establishment: a very promising aspect in view of their application under agricultural conditions. Interestingly, CO did not anticipate symbiosis senescence, as suggested by the arbuscule regular morphology in both treated and untreated samples. The fact that the longer time-span of AM colonization in CO-treated plants did not correlate with a higher P content could appear surprising. Nevertheless, unchanged P contents are also reported in studies on hypermycorrhizal mutants of *M. truncatula* (Truong et al., 2014) and *Pisum sativum* (Jones et al., 2015), where arbuscule proliferation appears analogous to what we have observed upon CO treatment. A few hypotheses can be made to explain this, each of which deserves further investigation. Firstly, P uptake might not be the main function of the morphologically active arbuscules that we have observed at 28 dpi. Under our standard growth conditions, fewer arbuscules - as in untreated mycorrhizal plants - might be sufficient to grant *M. truncatula* an optimal P nutrition. The plant might instead take advantage of such an abundant number of arbuscules for other functions, such as water uptake. Challenging CO-treated and untreated plants with different levels of drought will shed light on this possibility, but our observation of a larger biomass in CO-treated mycorrhizal plants at 48 dpi - when the root system had extended to the whole pot volume – hints at a better performance of this experimental line under incipient stress conditions.

Secondly, prolonged CO elicitation could stimulate sustained fungal accommodation, while arbuscule functioning and senescence could be regulated by different - and yet unexplored - signaling processes. Lastly, one cannot exclude that such an abundant root colonization in a mature stage of the symbiosis is favoring the AM fungus absorption of plant-derived organic compounds, with CO elicitation possibly weakening the plant control mechanisms on over-colonization. Under this respect, it will be extremely important to investigate C flux toward the fungus, as well as extraradical mycelium development or spore production.

362

363 *4.2. Plant responses to CO treatments*

364 The use of different chitin-derived molecules in agricultural practices or laboratory conditions is
365 not new. Benefits to the plant have been described in terms of better growth and resistance to biotic
366 or abiotic stresses. Nevertheless, a clear picture of their multiple effects on plants is far from being
367 understood and most agricultural applications rely on the heuristic observation of a correlation
368 between chitin-based treatments and beneficial effects. In this frame, our results provide a possible
369 explanation to such positive effects on crops: if such mixes of compounds include short-chain CO,
370 then part of the benefits to the plant may derive from its improved symbiotic status, which in turn is
371 well known to support plant development, defense and stress resistance.

372 In the field of plant-microbe interactions, the length of CO molecules is acknowledged as a major
373 determinant of plant responses, with the following - likely simplistic - scenario: chito-biose and -
374 triose are reported to produce little or no stimulation in plant cells; -tetraose and -pentaose have been
375 characterized as symbiotic signals in all AM host plants (Genre et al., 2013; Giovannetti et al, 2015;
376 Sun et al., 2015); whereas longer CO progressively shift toward defense elicitation (Liu et al., 2012;
377 Hayafune et al., 2014), with chito-octamers being the best characterized chitin-based pathogen-
378 associated molecular pattern (Zhang et al., 2002; Cao et al., 2017). In this frame, CO have been
379 proposed as primers of plant defenses in agriculture, even if limited attention has been put on the
380 oligomer composition of the mixture. The picture is even less clear about the effects of CO on plant
381 development. A stimulating effect of chito-pentaose was demonstrated on Arabidopsis growth (Khan
382 et al., 2011). Similarly, Winkler and colleagues (2017) demonstrated that a mixture of short-chain
383 CO, increased Arabidopsis and poplar shoot explant development, and chito-tetraose treatment of
384 Arabidopsis seedlings upregulated development, cell organization, biogenesis and transport-related
385 genes. By contrast, no stimulating effect on plant growth was observed for CO by Chatelain et al.
386 (2014), who rather reported a decrease in *Phaseolus vulgaris* biomass. Zhang et al (2016) indicated

CO length as a critical determinant of their effect also on plant development, with chito-hexaose and -heptaose inducing the strongest elicitation of biomass development in wheat.

This complex scenario is difficult to compare with our experimental system, based on CO treatment of mycorrhizal plants. Nevertheless, a few parallels may be drawn. The reduction of uninoculated plant development observed in CO irrigated plants, appears in line with the inhibition of plant growth described by Zhang et al. (2016), since we were using a mix of CO that did not include the growth-promoting hexa- and heptamers. By contrast, a synergistic positive effect of CO and inoculation on plant development was observed near the end of our experimental time frame. In this case, fungal presence is introducing an additional level of complexity to the experimental system, complicating the interpretation of direct and combined effects of CO, symbiotic status and stress conditions possibly induced by the space limitation of the pot culture.

5. Conclusion

The introduction of AM as biofertilizers in sustainable agricultural practices has been proposed for decades as a promising natural solution to the increasing worldwide demand of food safety. Results have anyway been contrasting, with two major obstacles emerging. First, even if AM fungi have low host specificity, studies quickly highlighted that not all fungal inocula are suitable for all agricultural applications: local climate, soil characteristics, as well as the species (if not the variety) of the selected crop generate a matrix of variables that only customized inocula could address, with the time- and resource-consuming selection of performing fungal strains for each condition. Second, a partial recalcitrance has been observed in cultivated species toward AM interaction, likely due to decades of excessive use of fertilizers (Duhamel & Vandenkoornhuyse, 2013) and to farming practices that have always been attentive to the aerial part of the plant but unaware of its underground interplay.

Our study showed that CO treatments strongly enhance AM colonization, anticipating the upregulation of AM marker genes, but not arbuscule senescence. Even if several aspects of CO effects

412 on plants remain to be explored, our demonstration of their positive impact on AM establishment
413 represents a breakthrough on the way to the large scale introduction of AM in agricultural practices,
414 by positively acting on the mycorrhizal aptitude of the host plants.
415
416 **Acknowledgements**
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420
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569
570
571 **Figure legends.**
572 **Fig. 1.** Nuclear Ca^{2+} spiking in response to crustacean-derived CO. A) Representative plots of nuclear
573 Ca^{2+} variations over 30 minutes in atrichoblast nuclei of *M. truncatula* root organ culters (ROCs)
574 treated with water (control), 1mg/L and 1g/L CO. Both CO concentrations induced a series of Ca^{2+}
575 peaks (spiking), characteristic of AM fungal recognition. B) Statistical analyses of the spiking
576 response (t-Student test with $P < 0.05$) did not highlight significant differences between CO
577 treatments, in terms of either the percentage of responding atrichoblasts (top panel) or average peak
578 number in responding cells (bottom). Bars represent standard errors. At least 100 atrichoblasts from
579 seven independent root samples were analyzed for each experimental condition.
580
581 **Fig. 2.** Effect of spray CO treatment on mycorrhizal plants at 28 dpi (A) and 48 dpi (B). Images of
582 root sections of mycorrhizal plants untreated (Myc) and treated with CO (Myc+CO). A strong
583 proliferation of arbuscules (white dots) was evident in Myc+CO plants, with the colonization of
584 multiple cortical layers at both time points, as evident in the magnified areas (red square) in the lower
585 panels. This was confirmed by the quantitative analysis of root colonization, shown in the bottom
586 graphs: mycorrhization frequency (F), mycorrhization intensity (M), arbuscule abundance within

587 colonized root fragments (a) and arbuscule abundance in the whole root system (A) all reported
588 significantly higher values in Myc+CO compared to Myc plants. The effect of CO on root
589 colonization was anyway more evident at 28 dpi. Mean values and SDs of ten biological replicates
590 for each treatment are shown. Bars = 100 μm.
591
592 **Fig. 3.** Aboveground development in response to spray CO treatment. A) Average photosynthetic
593 surface (including representative scans of the plant epigeral parts) and shoot dry biomass at 28 dpi:
594 the photosynthetic surface was significantly more developed in Myc and Myc+CO plants compared
595 to Ctr and Ctr+CO; a similar – albeit statistically non significant - trend was observed for shoot dry
596 weight. B) The photosynthetic surface was significantly more development in Myc+CO plants at 48
597 dpi, when both mycorrhizal plants showed a significant increase of shoot dry biomass. Mean values
598 and SDs are presented for five and ten biological replicates from control and mycorrhizal plants,
599 respectively.
600
601 **Fig. 4.** Leaf nitrogen (N) status at 28 and 48 dpi. The chlorophyll/flavonoid ratio (Chl/Flav), or
602 Nitrogen Balance Index (NBI), is presented here as an indicator of leaf N content. NBI value was
603 significantly higher in both mycorrhizal lines (Myc and Myc+CO) than controls (Ctr and Ctr+CO) at
604 28 dpi. By contrast, NBI was significantly higher in all CO treated plants at 48 dpi. Mean values and
605 SDs are presented for five and ten biological replicates from control and mycorrhizal plants,
606 respectively.
607
608 **Fig. 5.** Time-course analysis of *MtBCP* and *MtPT4* expression. Asterisks indicate the significant early
609 induction of *MtBCP* at 7 dpi and the significant downregulation of both genes at 21 and 28 dpi in
610 CO-treated plants (Myc+CO). Average values from at least 5 biological replicates are presented.
611

612 **Fig. 6.** Molecular analysis on roots at 28 dpi after two CO treatments. Two of the three AM-induced
613 chitinases are strong induced in the mycorrhizal plants, but the transcripts levels for *MtChitIII.2* and
614 *MtChitIII.4* are significant reduced in the Myc+CO compared to the Myc untreated plants, in line
615 with the expression profile of *MtPT4*. Mean values and SDs in three biological replicates for each
616 treatment are shown.
617
618 **Fig. 7.** Phosphorous (P) content level in untreated (Myc) and treated (Myc+CO) mycorrhizal plants.
619 Both lines reached the highest leaf P content at 14 dpi but no significant difference was detected in
620 any time point. Mean values for five biological replicates are shown. Bars represent standard errors.
621
622 **Fig. 8.** Expression of pathogenesis-related genes in roots at 28 dpi after two CO treatments. The three
623 analysed pathogenesis-induced genes, *MtChitI*, *MtChitIII.1* and *MtPR10*, were not upregulated upon
624 treatment with crustacean-derived CO, suggesting that they were not perceived as a pathogenesis
625 signal. Mean values and SDs in three biological replicates for each treatment are shown.
626
627 **Supplementary data**
628 **Fig. S1.** Composition of crustacean-derived CO identified by HPLC-MS/MS. CO molecules were
629 detected with a chain length ranging between 2 and 5 residues and different degrees of deacetylation.
630 A) Since CO detection threshold raises with increasing chain length, only the presence (+) or absence
631 (-) of each type of molecule is presented here. NS = not searched. B) Structure of mono-deacetylated
632 (beta anomer) CO4 and theorical precursor/product ions forming in LC-MS/MS the positive mode.
633 C) LC-MS/MS chromatogram in the MRM mode (M+H+). The two peaks correspond to alpha and
634 beta anomers of the molecule.
635
636 **Fig. S2.** Schemes representing the experimental set-up for CO treatment and sampling used for this

work. The camera symbol indicates photographic recording of plant development; dpi, days post inoculation; dpt, days post transplant.

Fig. S3. Monitoring of plant growth during CO spray treatment. The growth of control (Ctr), CO-treated control (Ctr+CO), mycorrhizal (Myc) and CO-treated mycorrhizal plants (Myc+CO) was monitored at 7, 28 and 48 dpi. AM inoculation alone increased plant growth at both 28 and 48 dpi, but aerial part development at both time points was further enhanced when AM inoculation was combined with CO application (Myc+CO) at 14, 21, 28, 34, 40 and 46 dpi as summarized in Fig. S2.

Fig. S4. Effect of CO treatment by irrigation on plant development at 48 dpi. Shoot and root biomass production was monitored in control (Ctr), CO-treated control (Ctr+CO), mycorrhizal (Myc) and CO-treated mycorrhizal plants (Myc+CO). A reduced development of both shoots and roots was observed in Ctr+CO compared to Ctr plants. While root biomass was not affected by AM inoculation, Myc and Myc+CO plants displayed an increased shoot biomass production compared to Ctr, with consistent results for fresh weight, dry weight and photosynthetic surface. Mean values and SDs of ten biological replicates for each treatment are shown.

Fig. S5. Effect of CO treatment by irrigation on mycorrhizal development at 48 dpi. Images of root sections from untreated (Myc) and CO-treated mycorrhizal plants (Myc+CO). Arbuscules proliferation was strongly stimulated in Myc+CO plants, with the involvement of multiple cortical layers, compared to a more limited colonization, surrounding the vascular cylinder (vc), in Myc plants. This observation was confirmed by the quantitative analysis of root colonization, reported in the bottom graph: mycorrhization frequency (F), mycorrhization intensity (M), arbuscule abundance within colonized root fragments (a) and arbuscule abundance in the whole root system (A) all reported significantly higher values in Myc+CO compared to Myc plants. Mean values and SDs of ten

biological replicates for each treatment are shown. Bars = 200 µm

Fig. S6. Arbuscule phenotype in Myc and Myc + CO plants. In both cases, fully developed and highly branched arbuscules were observed at 28 dpi. Twenty root colonized sections from five biological replicates were used for each treatment. Bars = 30 µm.

Table S1. Primers used for qRT-PCR analysis

Figure 1

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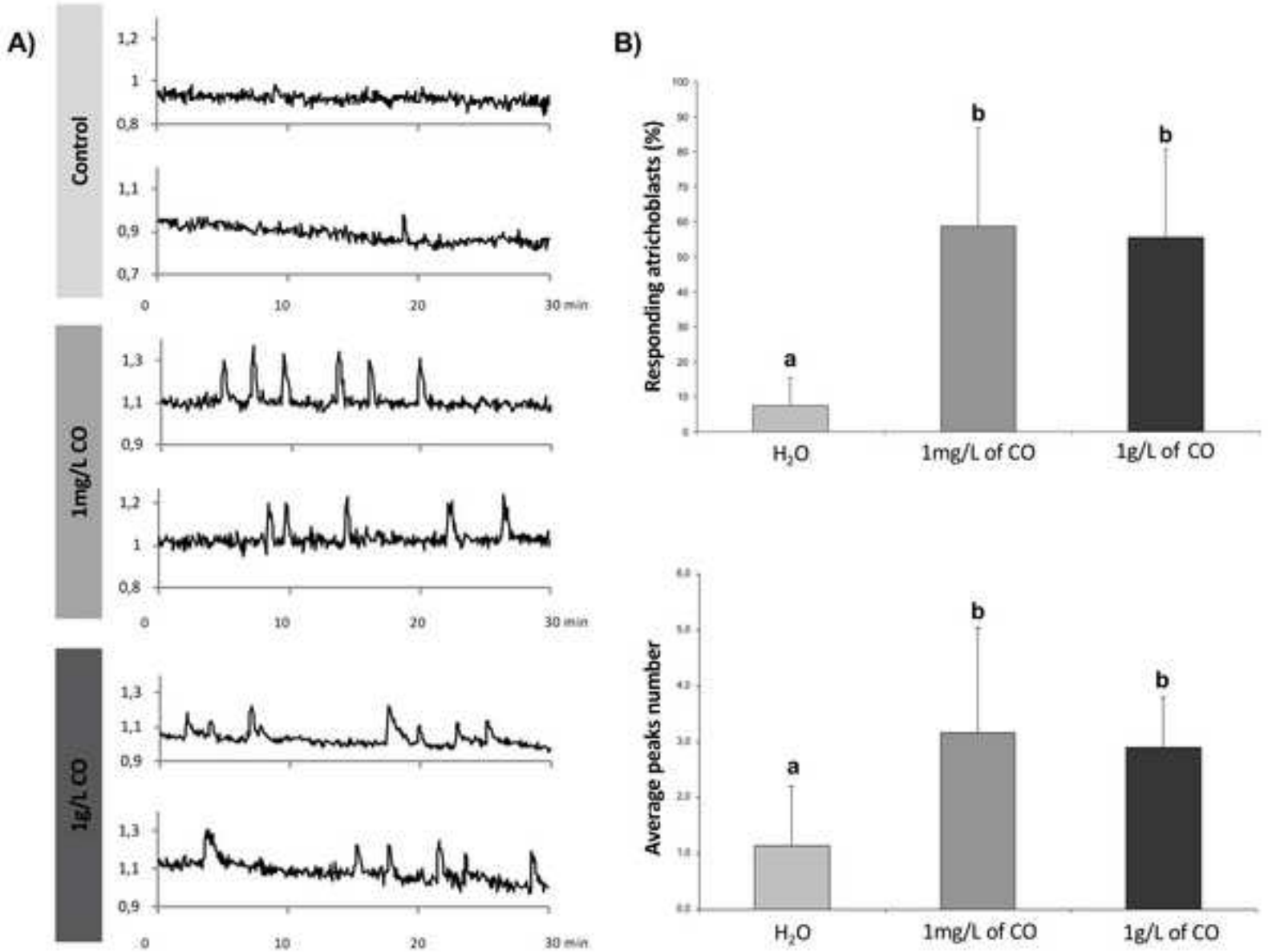

Figure 2

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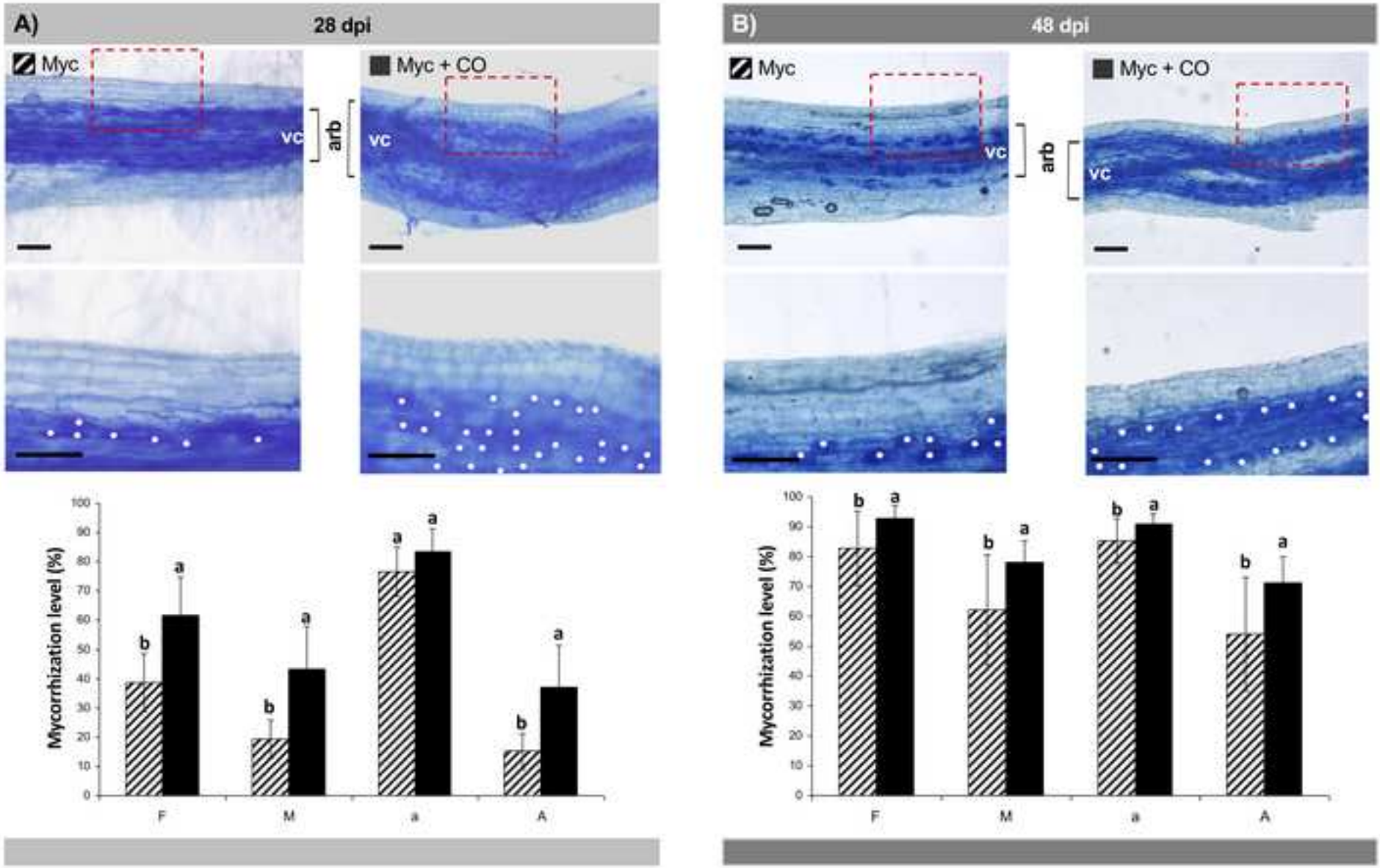

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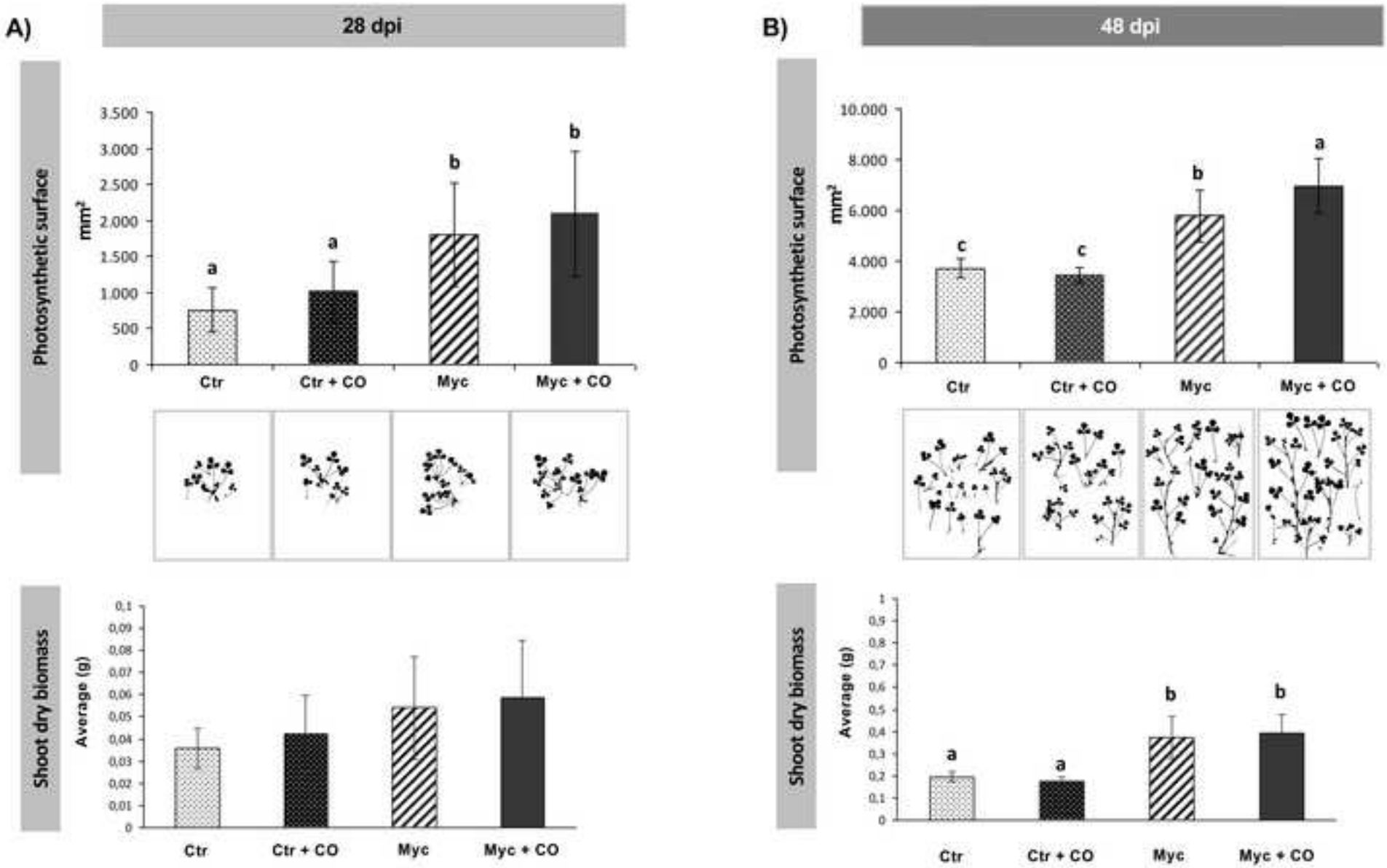

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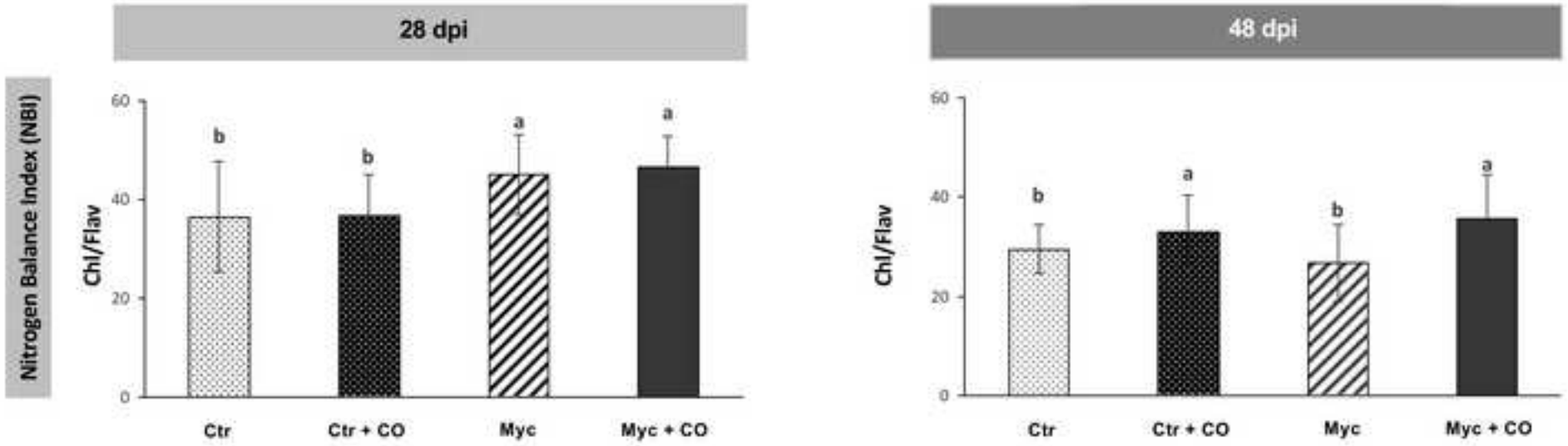

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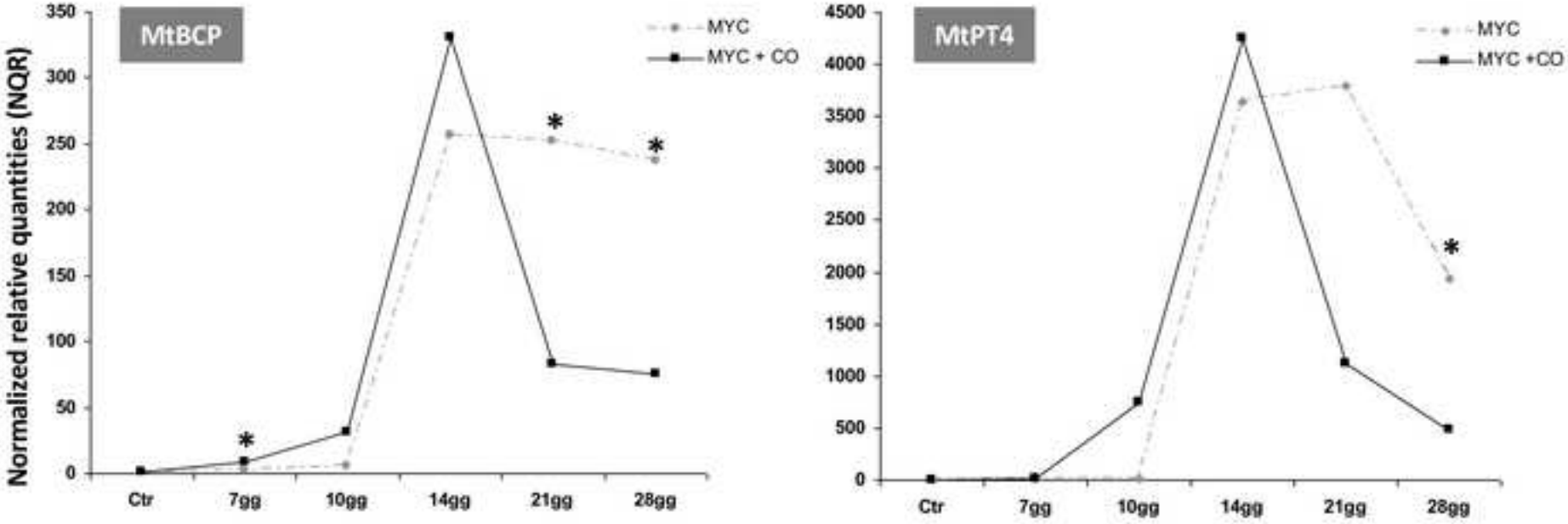

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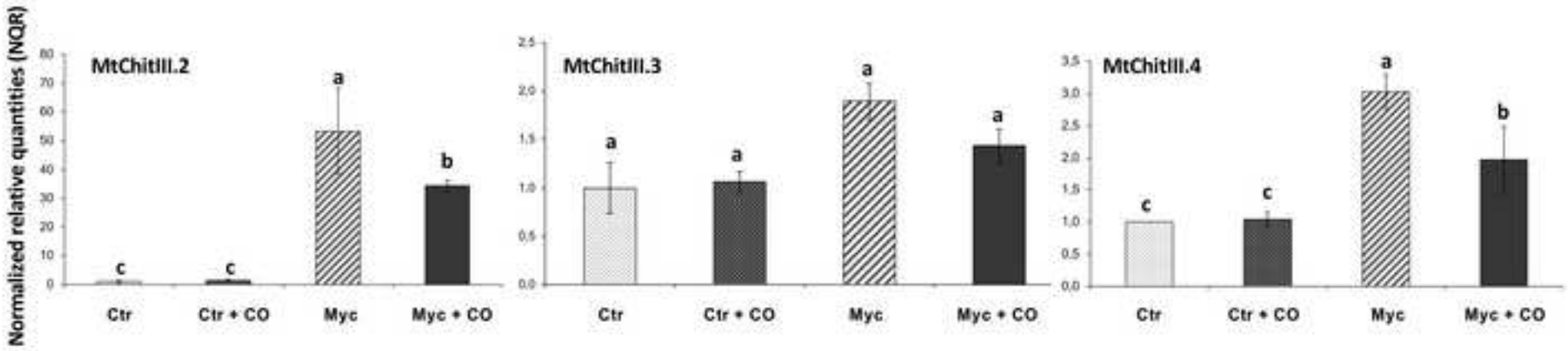

Figure 7
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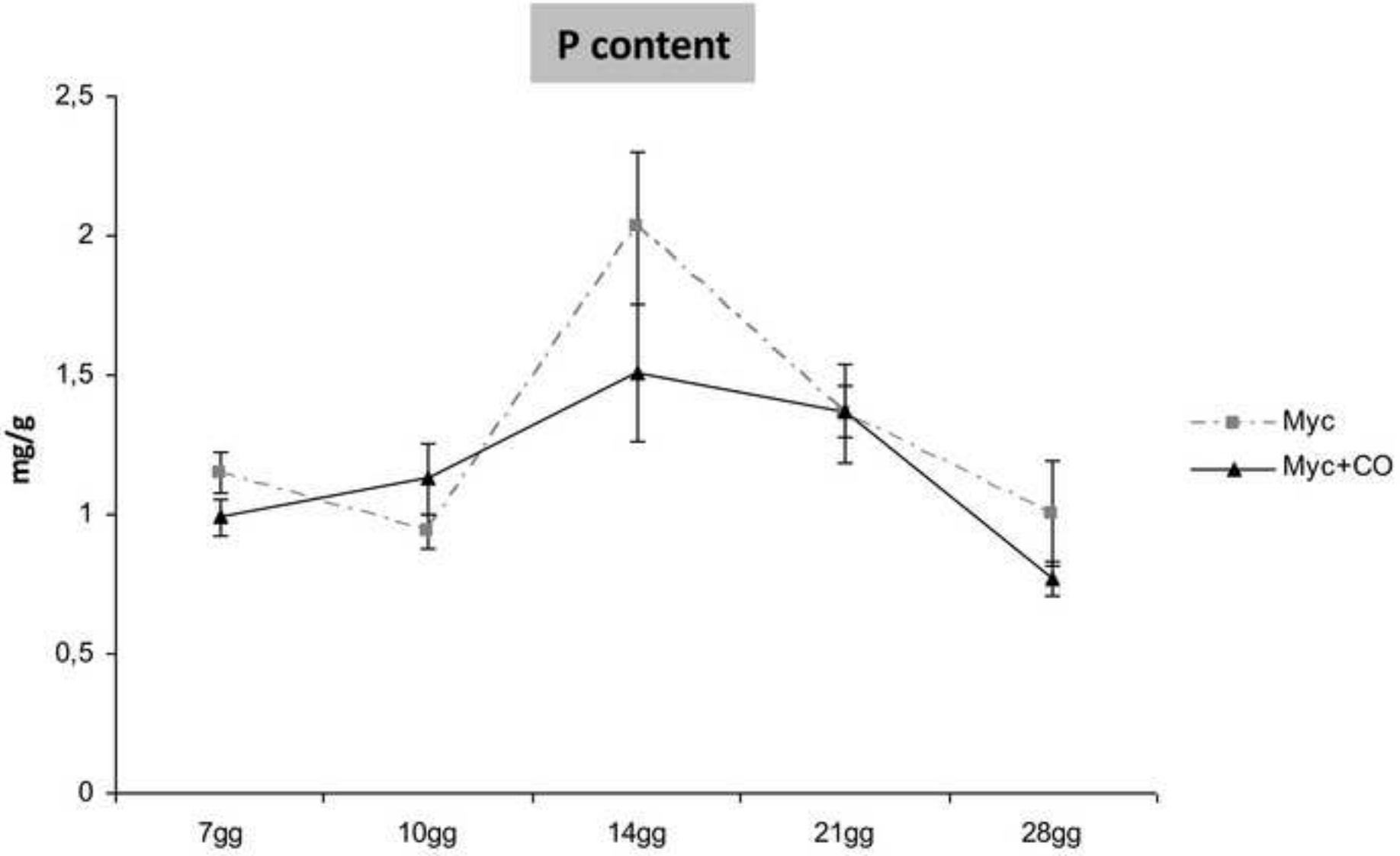

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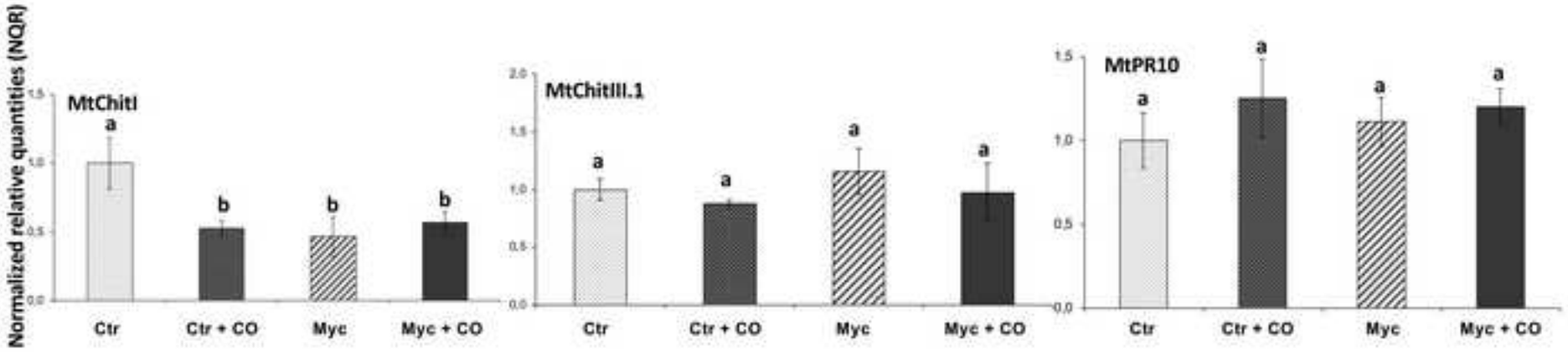

Table S1

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